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# DETECTION OF DIFFERENT STRAINS OF DIARRHEAGENIC ESCHERICHIA COLI FROM STOOL OF UNDER 5 CHILDREN WITH ACUTE DIARRHEA BY MULTIPLEX PCR IN CHATTOGRAM

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## ABSTRACT

**Background:** Diarrhaegenic *Escherichia coli* is one of the most important etiologic agent of acute diarrhea of under 5 children and represent a major public health problem in developing countries like Bangladesh. Due to lack of facilities diarrhaegenic*Escherichia coli* can not be detected in the routine diagnostic microbiology laboratory. Recently various multiplex PCR methods have been developed to detect diarrheagenic*Escherichia coli*. **Objective:** The aim of this study is to detect different diarrheagenic *Escherichia coli* strains (EPEC, ETEC and EAEC) by multiplex PCR. **Method:** The present study was conducted in the Department of Microbiology, Chattogram Medical College to detect different diarrheagenic*Escherichia coli* strains by multiplex PCR. This cross sectional study was done during the period from January 2017 to December 2017 and included under 5 children with acute diarrhea irrespective of sex admitted in Chattogram Medical College Hospital and Chattogram Maa-O-Shishu Hospital Medical College. A total of 250 stool samples were examined by standard laboratory methods for identification of bacterial enteropathogens. Different diarrheagenic*Escherichia coli* strains were detected by MultiplexPCR following standard methods**. Result:** Out of 250 diarrheal stool samples, diarrheagenic *Escherichiacoli* (DEC) were detected in 41 (16.4%) which included single strain of Enteropathogenic*E.coli* (EPEC) in 19 (46.34%) cases, single strain of Enteroaggregative*E coli* (EAEC) in 10 (24.39%) cases, single strain of Enterotoxigenic*E.coli* (ETEC) in 9 (21.95%) cases and combined strain of EPEC and EAEC in 3 (7.31%) cases. Other enteropathogens included *shigella spp.* in 23 (9.2%) and *salmonella spp*. in 15 (6%) cases. **Conclusion:** Analyzing the findings of this present study, diarrheagenic*Escherichia coli* was found to be one of the most important cause of acute diarrhea among under 5 children. Detection rate of diarrheagenic *Escherichia coli* by multiplex PCR method was quite satisfactory. Thereby, Multiplex PCR could be adapted to detect different diarrheagenic*Escherichia coli* in setup like Medical colleges or tertiary medical facilities.

**KEYWORDS:** Diarrhaegenic Escherichia, multiplex PCR, acute diarrhea.

## INTRODUCTION

Diarrhea is defined by World health organization (WHO) as having 3 or more loose or liquid stool per day or as having more stool than in normal for that person. Diarrhea is a leading killer of children, accounting for approximately 8 per cent of all deaths among children under age 5 worldwide in 2016. This translates to over 1,300 young children dying each day, or about 480,000 children a year, despite the availability of simple effective treatment (WHO, 2017).[1]

*Escherichia coli* is the predominant facultative anaerobe of the human colonic flora. *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. In *Escherichia coli* strains, there are several highly adapted clones that have the capacity to cause human illness. Strains that cause enteric infections are designated diarrheagenic *E. coli*, a group that includes emergent pathogens with public health relevance worldwide.[2] Each type of *E. coli* diarrhea is associated with a different pathotype of *E. coli* and each pathotype has characteristic virulence determinants that contribute to its pathogenic mechanisms.[3]

Diarrheagenic Escherichia coli (DEC) cause acute and persistent diarrhea worldwide.[4] Diarrheagenic *E. coli* may be an important and unrecognized cause of diarrhea in infancy, not only in developing countries but also in developed areas (Amisano et al, 2011).[5] In Bangladesh, 34% DEC were identified by multiplex PCR among diarrheic patients under 5 years of age in one study (Roy et al.,2014).[6]

Due to lack of facilities, DEC can not be detected in the routine diagnostic microbiology laboratory in developing countries, which is important in understanding the disease spectrum, tracing the sources of infection and routes of transmission and understanding the burden of the disease. Such identification would also assist the clinician to dispense appropriate management (Nessa et al., 2007).[7]

To correctly identify diarrheagenic *E. coli* strains, these organisms must be differentiated from nonpathogenic members of the normal flora. Serotypic markers correlate, sometimes very closely, with specific categories of diarrheagenic *E. coli*; however, these markers are rarely sufficient in and of themselves to reliably identify a strain as diarrheagenic. Thus, the detection of diarrheagenic *E. coli* has focused increasingly on the identification of certain characteristics which themselves determine the virulence of these organisms. This identification process may include HEp-2 cell adherence, DNA hybridization, and PCR assays to detect the presence of specific virulence traits or the genes encoding these traits. The first two types of assays require special expertise, employ cell culture and radioactive material, and are time-consuming. However, more researchers are using molecular methods such as polymerase chain reaction (PCR) to identify pathotypes. A PCR assay based on identifying the presence of specific virulence genes, which are absent in nonpathogenic strains, may provide a more efficient way to diagnose DEC strains in fecal samples (Aranda et al., 2004).[8]

## OBJECTIVE

### General objective

To detect different diarrheagenic *Escherichia coli* strains (EPEC, ETEC and EAEC) by multiplex PCR.

### Specific objectives

1. To isolate and identify *Escherichia coli* by stool culture and different biochemical tests.
2. To find out the frequency of different strains of diarrheagenic *Escherichia coli* by multiplex PCR.
3. To find out antimicrobial sensitivity patterns of the isolated DEC strains.

## METHODOLOGY

### Study Design

The study was descriptive type cross-sectional study.

**Study place** The study was carried out in the department of Microbiology of Chattogram Medical College Hospital, Chattogram.

### Duration of study

Study period was carried out from 1st january, 2017 to 31st december, 2017.

### Study population

Children under 5 years of age with acute diarrhea attending outdoor and indoor of Chattogram Medical College Hospital and Chattogram Maa-O-Shishu Hospital Medical college was enrolled in this study.

### Sample size

Minimum sample size is 344. But in our study, sample size is 250 due to limitation of resources and time.

### Selection criteria

### Inclusion criteria

Children under 5 years of age with following criteria
At least 3 times passage of loose or watery stool within 24 hours.

### Exclusion criteria

Chronic diarrhea (diarrhea more than 14 days).

### Data collection

Data collection was done by using structural questionnaire and checklist.

### Data analysis

Data was collected and recorded in a predesigned data sheet The results of the experiments were recorded systematically and statistical analysis was done by standard statistical procedure.

### Sampling technique

Non-probability purposive type of sampling.

## RESULTS

**Table 1:** Shows age and sex distribution of the patients. The age of the patients varied from 6 months to 60 months as no samples were found from 0-6 months of age group. Among the study population, male were 151 and female were 99.The male to female ratio was 1.5:1 and the difference was not statistically significant ($p>0.05$).

**Table 1: Age and sex distribution of the patients (n=250).**

| Age group (Months) | Sex | | Total |
|---|---|---|---|
| | Male | Female | |
| 6 m-12 m | 73 (29.2%) | 39 (15.6%) | 112 (44.8%) |
| 13 m-24 m | 34 (13.6%) | 25 (10.0%) | 59 (23.6%) |
| 25m-36 m | 20 (8.0%) | 15 (6.0%) | 35 (14.0%) |
| 37m-48 m | 8 (3.2%) | 8 (3.2%) | 16 (6.4%) |
| 49m-60 m | 16 (6.4%) | 12 (4.8%) | 28 (11.2%) |
| **Total** | **151 (60.4%)** | **99 (39.6%)** | **250 (100%)** |

$\lambda^2$= 2.262, df=4, p=0.688

**Table 2:** Shows the distribution of different culture isolates. A total of 250 children with acute diarrhea were studied and *E*. coli were isolated by stool culture from 185 (74%) samples. Among other organism, *Shigella* spp was 23 (9.2%), *Salmonella* spp was 15 (6%), *Klebsiella* spp was 24 (9.6%), *Proteus* was 2 (0.8%) and *Enterobacter* was 1 (0.4%).

**Table 2: Distribution of different culture isolates (n=250).**

| Isolates | Frequency (%) |
|---|---|
| *E. coli* | 185 (74%) |
| *Shigella* spp | 23 (9.2%) |
| *Salmonella* spp | 15 (6%) |
| *Klebsiella* spp | 24 (9.6%) |
| *Proteus* spp | 2 (0.8%) |
| *Enterobacter* spp | 1 (0.4%) |
| **Total** | **250 (100%)** |

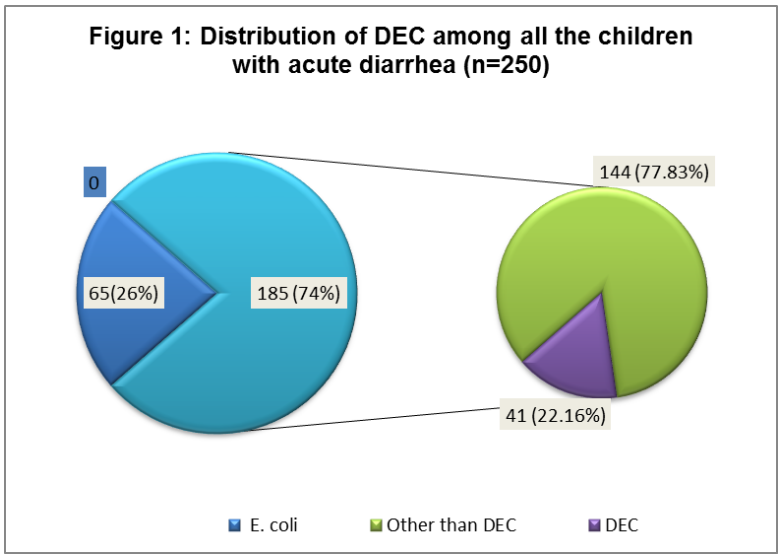


**Figure I: Shows distribution of DEC in study population. 185 (74%) *E. coli* was isolated among 250 diarrheic children. Multiplex PCR was done on this isolated *E. coli* and 41 (22.16%) DEC was isolated among them.**

Table 3: Frequency of the isolated different strain and their virulence gene among the total isolated DEC are shown in Table 3. 41samples of the patients 16.4% (41/250) had been positively recognized for genes related to pathogenesis: 38 were single strain and 3 were combined strain of DEC. 19 (46.34%) were single strain of EPEC (all EPEC with the *eae* genes, no *bfp* gene were found), 10 (24.39%) were single strain of EAEC with *aat* gene, 9 (21.95%) were single strain of ETEC [7 (17.06%) were atypical ETEC (4 with *st* and 3 with *It* gene), 2 (4.87%) were typical ETEC (both with the *lt* and *st* genes] and 3 (7.31%) were combined strain of EPEC+EAEC with *eae+aat* gene.

**Table 3: Frequency of the isolated different strain and their virulence gene among the total isolated DEC (n=41).**

| Isolated strain | Virulence gene | No. of the patients |
|---|---|---|
| EPEC<br>19 (46.34%) | *eae*<br>*bfp* | 19 (46.34%)<br>0 (0%) |
| EAEC<br>10 (24.39%) | *aat* | 10 (24.39%) |
| ETEC<br>9 (21.95%) | *st*<br>*lt*<br>*st+lt* | 4 (9.75%)<br>3 (7.31%)<br>2 (4.87%) |
| EPEC+EAEC<br>3(7.31%) | *eae+aat* | 3 (7.31%) |

**Table 4:** Shows the age distribution of the diarrheic patients with respect to incidence of different DEC strains. EPEC (21.95%) was found frequent in the children of 6-12 month age group, EAEC (9.75%) was found frequent in 13-24 month age group, ETEC (9.74%)was found frequent in the children of 6-12 month age group ,combined strain of EPEC+EAEC was found only in 6-12 month age group . The differences in distribution among different age group was not statistically significant (p>0.05).

**Table 4: Distribution of the isolated strain by age group of the patients.**

| Strain | 6-12m | 13-24 m | 25-36 m | 37-48m | 49-60m | Total |
|---|---|---|---|---|---|---|
| EPEC | 9(21.95%) | 5(12.19%) | 2(4.87%) | 1(2.43%) | 2(4.87%) | 19(46.34%) |
| EAEC | 2(4.87%) | 4(9.75%) | 2(4.87%) | 1(2.43%) | 1(2.43%) | 10(24.39%) |
| EPEC+EAEC | 3(7.31%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 3(7.31%) |
| ETEC(Atypical) | 3(7.31%) | 2(4.87%) | 1(2.43%) | 1(2.43%) | 0(0%) | 7(17.07%) |
| ETEC(Typical) | 1(2.43%) | 0(0%) | 1(2.43%) | 0(0%) | 0(0%) | 2(4.87%) |
| **Total** | 18(43.90%) | 11(26.82%) | 6(14.63%) | 3(7.31%) | 3(7.31%) | 41(100%) |

$\lambda^2$= 10.367, df=16, p=0.847

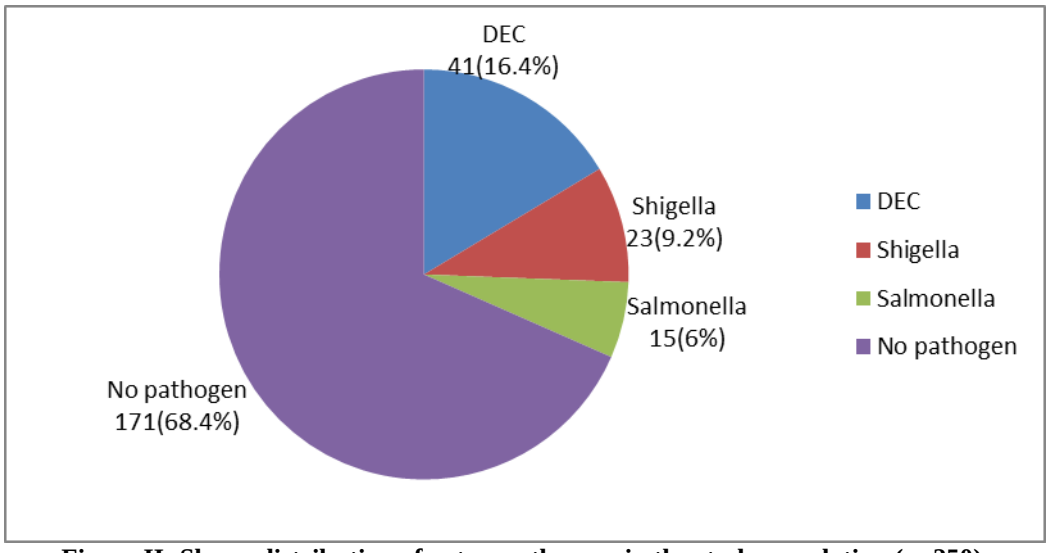


**Figure II: Shows distribution of enteropathogens in the study population (n=250).**

Among 250 diarrheal children, DEC was isolated in 41 (16.4 %) cases by multiplex PCR, *shigella* in 23 (9.2 %) cases, *salmonella* in 15 (6 %) Cases and no pathogen was found in 171 (68.4 %) cases which included non pathogenic *E. coli* – 144 (57.6%), *klebsiella*-24 (9.6%), *proteus-2 (0.8%)* and *Enterobacter* -1 (0.4%).

Table 5: Shows antibiotic sensitivity pattern of single strain of diarrheagenic *E. coli*:

**EPEC strain:** Antibiotic sensitivity pattern of DEC were tested against twelve antibiotics. Most of the EPEC strains of diarrheagenic *E.* coli were sensitive to cefuroxime, ceftriaxone and mecillinam (84.21%) followed by cefixime (78.94%); gentamycin (68.42%); ceftazidime (73.68%); ciprofloxacin (57.80%); azithromycin (47.36%); cotrimoxazole and nalidixic acid (26.31%); erythromycin (21.05%) and ampicillin (10.52%).

**EAEC strain:** Most of the EAEC strains of diarrheagenic *E. coli* (90%) were sensitive to cefuroxime, ceftriaxone, and mecillinam followed by cefixime and gentamycin (80%); erythromycin (70%);

ceftazidime, azithromycin and ciprofloxacin (60%); co-trimoxazole (40%); nalidixic acid (30%) and ampicillin (0%).

**ETEC strain** Most of the ETEC strains (88.9%) were sensitive to ceftriaxone followed by mecillinam (78%); cefuroxime (77.77%); gentamycin, cefixime and ciprofloxacin (66.7%); azithromycin and ceftazidime (55.55%); cotrimoxazole and erythromycin (44.44%); nalidixic acid (33.33%) and ampicillin (22.22%).

**Table 5: Antibiotic sensistivity pattern of sample containing single strain of diarrheagenic *E. coli.***

| Antibiotics | EPEC (n=19) | | EAEC (n=10) | | ETEC (n=9) | |
|---|---|---|---|---|---|---|
| | S | R | S | R | S | R |
| Mecillinam | 16 (84.21%) | 3 (15.79%) | 9(90%) | 1 (10%) | 7(77.77) | (23.30%) |
| Gentamycin | 13 (68.42%) | 6 (31.58%) | 8(80%) | 2 (20%) | 6(66.66) | 3(33.34%) |
| Azithromycin | 9 (47.36%) | 10 (52.64%) | 6(60%) | 4 (40%) | 5(55.55) | 4 (44.45%) |
| Cotrimoxazole | 5 (26.31%) | 14 (73.69%) | 4(40%) | 6 (60%) | 4(44.45%) | 5 (55.55%) |
| Erythromycin | 4 (21.05%) | 15 (78.95%) | 7(70%) | 3 (30%) | 4(44.45%) | 5 (55.55%) |
| Ampicillin | 2 (10.52%) | 17 (89.48%) | 0 (0%) | 10(100%) | 2(22.22%) | 7 (87.88%) |
| Cefixime | 15 (78.94%) | 4 (21.05%) | 8(80%) | 2 (20%) | 6(66.66%) | 3 (33.34%) |
| Cefuroxime | 16 (84.21%) | 3 (15.79%) | 9(90%) | 1 (10%) | 7(77.77%) | 2 (23.30%) |
| Ceftazidime | 14 (73.68%) | 5 (26.31%) | 6(60%) | 4 (40%) | 5(55.55%) | 4 (44.45%) |
| Ceftriaxone | 16 (84.21%) | 3 (15.79%) | 9(90%) | 1 (10%) | 8(88.88%) | 1 (11.12%) |
| Ciprofloxacin | 11 (57.89%) | 8 (42.11%) | 6(60%) | 4 (40%) | 6(66.66%) | 3 (33.34%) |
| Nalidixic acid | 5 (26.31%) | 14 (73.69%) | 3(30%) | 7 (70%) | 3(33.34%) | 6 (66.66%) |

## DISCUSSION

In the present study, majority of the cases 112 (44.8%) belonged to 6-12 month age group, 59 (23.6% ) cases were found in 13-24 month, 35 (14%) cases were found in 25-36 month, 28 (11.2%) cases were found in 49-60 month and 16 (6.4%) in 37-48 month age group (Table:1). The age of the patients varied from 6 months to 60 months as no samples were found from 0-6 months of age group. In one study, most diarrheic children (52.0%) were less than 24 months[9]. Vilchez et al. (2009) found 53.2% diarrheic children below 12 months age group.[10] Out of 250 cases, 151 (60.4%) were male and 99 (39.6%) were female. The male and female ratio was 1.5:1. Similar findings of male dominance over female cases were reported in another study in Bangladesh where male and female ratio was 1.3:1.

Out of 250 diarrheal stool samples, *E. coli* were isolated by culture from 185 (74%) (Table: 2). 67.5% *E. coli* were isolated from stool of under 5 diarrheal children in one study in Bangladesh (Roy et al., 2014). The isolation rate of *E. coli* from diarrheal stool sample was 60.9% in another study.[11] Among other culture isolated organism, *Shigella* spp was 23 (9.2%), *Salmonella* spp was 15 (6%), *Klebsiella* spp was 24 (9.6%), *Proteus* spp was 2 (0.8%) and *Enterobacter* spp was 1 (0.4%) in which last three spp were regarded as fecal flora (Table:2). *Shigella spp* was found to be the second most bacteria in the present study and the detection rate was 9.2% (23/250). Vargas et al. (2015) found 9% *shigella spp.*[4] *Shigella spp* was found 10% in one study in Bangladesh (Hossain et al., 2013)[12] In another study in Bangladesh, *Shigella spp* detection rate was found 7.6% in diarrheal patients (Hasan et al.,2006)[13] In the present study, *Salmonella spp* was found 6%. Jafari et al. (2009) found 7.5% *salmonella spp*.[14] In previous studies in Bangladesh, *Salmonella spp* detection rate was 1.83%-10% among diarrheal patients.

Among the bacterial pathogens, diarrheagenic *E. coli* (16.4%, 41/250) was the most prevalent in our study population (figure II). 18.4% DEC were isolated among under 5 children in Nigeria.[15] One study reveals the incidence of DEC as an etiological agent of diarrhea upto a level of 21%.[16] DEC strains were detected in 23.3% cases in Mexico.[17]

In this study, 19 (46.34%) were single strain of EPEC (all EPEC with the *eae* genes, no *bfp* gene were found), 10 (24.39%) were single strain of EAEC with *aat* gene, 9 (21.95%) were single strain of ETEC [7 (17.07%) atypical ETEC (4 with *st* and 3 with *It* gene), 2 (4.87%) typical ETEC (both with the *lt* and *st* genes ] and 3 (7.31%) combined strain of EPEC and EAEC with *eae+aat* gene (Table: 3).

In the present study, single strain of EAEC were found in 10 (24.39%) cases. 24% EAEC was found by Vargas et al. (2015).[4] EAEC accounted for 26.47% in previous study in Bangladesh. All the above study findings correlate well with the present study findings.

In the present study, among 41 DEC positive cases, 18 (43.9%) and 11 (26.8%) were from 6 to 12 and 13 to 24 months of age groups respectively and this difference was not statistically significant ($p>0.05$) (Table: 4). In one study, 64.5% DEC were characterized from children less than 1 year old. Another study reveals that 23.8% DEC occurred below two years of diarrheal children.[9] Most of the DEC strains (30.4%) were detected in under 2 years of age and 14-25.5% were found in other age group.[17] The proportion of *E. coli* positive for any intestinal pathotype was 18.6% in children less than 2

years of age and only 7.5% in children above 2 years.[18] In our study, EPEC (21.95%) was found frequent in the children of 6-12 month followed by 12.20% in 13-24 month age group (Table:4). EPEC infection is primarily a disease of infants younger than 2 years of age. Majority of cases of ETEC occur in children less than 2 years of age which also correlates with our study.[19]

In recent years, antibiotic resistance of diarrheagenic pathogens has reached alarming proportions worldwide. In the present study, resistance to ampicillin and nalidixic acid was shown by 89.48% and 73.69 % EPEC, 100 % and 70 % EAEC and 87.88 % and 66.66 % ETEC respectively (Table:5). In one study, resistance to ampicillin and nalidixic acid was found by 96% and 75% EPEC, 73% and 64% ETEC and 100% and 28% EAEC respectively which correlates with the present study.[20] Similarly, in Thailand isolated DEC showed high resistance to commonly used antibiotics such as ampicillin and nalidixic acid.[21] In this study, gentamicin was more effective than azithromycin showing resistance 31.58% by EPEC, 20% by EAEC and 33.34 % by ETEC. Regarding the aminoglycosides-gentamicin, low levels of intermediate resistance were found, collaborating data in the literature which suggest a good activity of these antimicrobials against enteric gram-negative bacilli. In our study, resistance to co-trimoxazole was shown by 73.69% EPEC, 60% EAEC and 55.55% ETEC. The levels of resistance observed for co-trimoxazole reflect the results from several studies by other authors who demonstrated high rates of resistance towards enteric *E. coli* against this drug. One explanation for this could be its widespread use in the treatment of diseases associated with gram-negative bacteria, especially in children under two years of age with acute infectious diarrhea (Roy et al., 2013)[20] In this study ciprofloxacin was resistant to 42.11% EPEC, 40% EAEC and 33.34% ETEC. The literature has reported varying rates of resistance against ciprofloxacin, which can be explained by the high prescription of this drug in some countries as a treatment for enteric infections caused by gram-negative bacteria (Livermore et al.,2002; Yang et al.,2009).[22,23]

## CONCLUSION

In conclusion, diarrheagenic *Escherichia coli* was found to be one of the most important cause of acute diarrhea among under 5 children in Chattogram city of Bangladesh. The development of multiplex PCR method for the simultaneous detection of several pathogenic genes in one PCR reaction will save time and effort involved in analyzing various virulence factors and will help investigators to clarify the role of diarrheagenic *E. coli* in diarrheal diseases.

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